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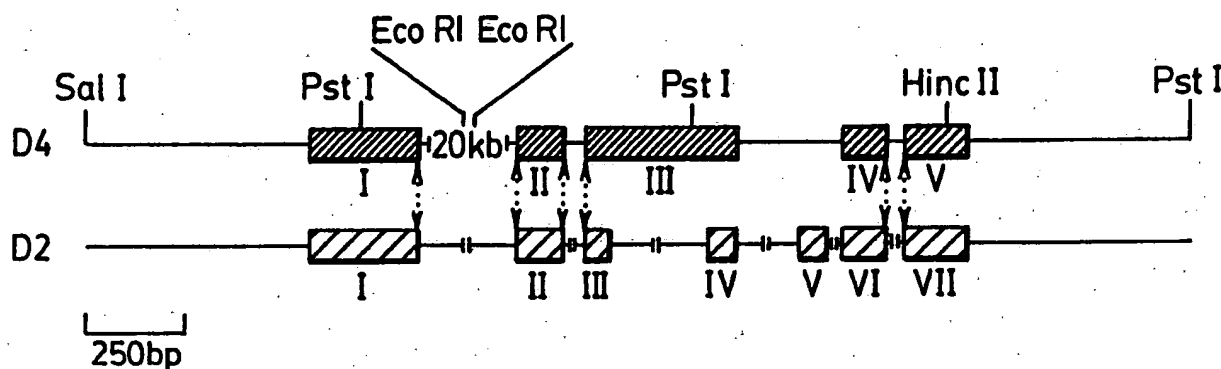
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| <p>(21) International Application Number: PCT/US91/09308<br/>(22) International Filing Date: 6 December 1991 (06.12.91)<br/>(30) Priority data:<br/>626,618 7 December 1990 (07.12.90) US<br/>(71) Applicant: STATE OF OREGON, acting by and through THE OREGON STATE BOARD OF HIGHER EDUCATION on behalf of THE OREGON HEALTH SCIENCES UNIVERSITY [US/US]; 1431 Johnson Lane, Eugene, OR 97403 (US).<br/>(72) Inventors: CIVELLI, Olivier ; 3012 Northeast 24th Avenue, Portland, OR 97212 (US). VAN TOL, Hubert ; 133 Brooke Avenue, Toronto, Ontario M5M 2K3 (CA).</p> | <p>(74) Agent: McDONNELL, John, M.; Allegetti &amp; Witcoff, Ten South Wacker Drive, Chicago, IL 60606 (US).<br/>(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), NO, SE (European patent).<br/><br/>Published<br/>With international search report.<br/>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p> |                                                                                                                            |

(54) Title: HUMAN DOPAMINE D<sub>4</sub> RECEPTOR AND ITS USES



(57) Abstract

The present invention is directed toward the isolation, characterization and pharmacological use of the human D<sub>4</sub> dopamine receptor. The nucleotide sequence of the gene corresponding to this receptor is provided by the invention. The invention also includes a recombinant eukaryotic expression vector capable of expressing the human D<sub>4</sub> dopamine receptor in cultures of transformed eukaryotic cells and such cultures of transformed eukaryotic cells which synthesize the human D<sub>4</sub> dopamine receptor.

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+ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

## Human Dopamine D4 receptor and its uses

## BACKGROUND OF THE INVENTION

This invention was made with government support under NIMH grant MH-45614 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. Field of the Invention

The invention relates to dopamine receptors from mammalian species and the genes corresponding to such receptors. In particular, it relates to the human dopamine receptor D<sub>4</sub>. Specifically, the invention relates to the isolation, cloning and sequencing of the human D<sub>4</sub> receptor gene. The invention also relates to the construction of eukaryotic expression vectors capable of expression of the human D<sub>4</sub> dopamine receptor in cultures of transformed eukaryotic cells and the synthesis of the human D<sub>4</sub> dopamine receptor in such cultures. The invention relates to the use of such cultures of transformed eukaryotic cells producing the human D<sub>4</sub> dopamine receptor for the characterization of antipsychotic drugs.

2. Information Disclosure

Dopamine is a neurotransmitter that participates in a variety of different functions mediated by the nervous system, including vision, movement, and behavior. See generally Cooper, J. et al., The Biochemical Basis of Neuropharmacology, 161-195 (Oxford University Press, NY 3d Ed. 1978). The diverse physiological actions of dopamine are in turn mediated by its interaction with two of the basic types of G protein-coupled receptors: D<sub>1</sub> and D<sub>2</sub>, which respectively stimulate and inhibit the enzyme adenylyl cyclase. Kebabian, J. and Calne, D., Nature 277: 93-96 (1979). Alterations in the number or activity of these receptors may be a contributory factor in disease states such as Parkinson's disease (a movement disorder) and schizophrenia (a behavioral disorder).

A great deal of information has accumulated on the biochemistry of the D<sub>1</sub> and D<sub>2</sub> dopamine receptors, and methods have been developed to solubilize and purify these receptor proteins. See Senogles, S. et al., Biochemistry 25: 749-753 (1986); Sengoles, S. et al., J. Biol. Chem. 263: 18996-19002 (1988); Gingrich, J. et al., Biochemistry 27: 3907-3912 (1988); Gingrich, J. et al. (in press). The D<sub>1</sub> dopamine receptor in several tissues appears to be a glycosylated membrane protein of about 72 kDa. Amlaiky, N. et al., Mol. Pharmacol. 31: 129-134 (1987); Ninik, H. et al., Biochemistry 27: 7594-7599 (1988). The D<sub>2</sub> receptor has been suggested to have a higher molecular weight of about 90 - 150 kDa. Amlaiky, N. and Caron, M., J. Biol. Chem. 260: 1983-1986 (1985); Amlaiky, N. and Caron, M., J. Neurochem. 47: 196-204 (1986); Jarvie, J. et al., Mol. Pharmacol. 34: 91-97 (1988). Much less is known about a recently discovered additional dopamine receptor, termed D<sub>3</sub>. Sokoloff, P. et al. Nature 347: 146-151 (1990). Dopamine receptors are primary targets in the clinical treatment of psychomotor disorders such as Parkinson's disease and affective disorders such as schizophrenia. Seeman, P. et al. Neuropsychopharm. 1: 5-15 (1987); Seeman, P. Synapse 1: 152-333 (1987). The three different dopamine receptors (D<sub>1</sub>, D<sub>2</sub>, D<sub>3</sub>) have been cloned as a result of nucleotide sequence homology which exists between these receptor genes. Bunzow, J.R. et al. Nature 336: 783-787 (1988); Grandy, D.K. et al. Proc. Natl. Acad. Sci. U.S.A. 86: 9762-9766 (1989); Dal Toso, R. et al. EMBO J. 8: 4025-4034 (1989); Zhou, Q-Y. et al. Nature 346: 76-80 (1990); Sunahara, R.K. et al. Nature 346: 80-83 (1990); Sokoloff, P. et al. Nature 347, 146-151 (1990).

The antipsychotic clozapine is useful for socially withdrawn and treatment-resistant schizophrenics [Kane, J. et al. Nature 347: 146-151 (1990)], but unlike other antipsychotic drugs, clozapine does not cause tardive dyskinesia [Casey, D.E. Psychopharmacology 99: 547-553 (1989)]. Clozapine, however, has dissociation constants at D<sub>2</sub> and D<sub>3</sub> which are 3 to 30-fold higher than the therapeutic free concentration of clozapine in plasma

water [Ackenheil, M. et al. *Arzneim-Forsch* 26: 1156-1158 (1976); Sandoz Canada, Inc., Clozaril: Summary of preclinical and clinical data (1990)]. This suggests the existence of dopamine receptors more sensitive to the antipsychotic clozapine.

5           We have cloned and sequenced such a human dopamine receptor which we term D<sub>4</sub>. The dopamine D<sub>4</sub> receptor gene has high homology to the human dopamine D<sub>2</sub> and D<sub>3</sub> receptor genes. The pharmacological profile of this receptor resembles that of the D<sub>2</sub> and D<sub>3</sub> receptors but it has an affinity for clozapine which is  
10       tenfold higher. The present inventors envision that the D<sub>4</sub> dopamine receptor disclosed as this invention may prove useful in discovering new types of drugs for schizophrenia that like clozapine do not induce tardive dyskinesia and other motor side effects.

## SUMMARY OF THE INVENTION

The present invention is directed toward the isolation, characterization and pharmacological use of the human D<sub>4</sub> dopamine receptor, the gene corresponding to this receptor, a recombinant eukaryotic expression vector capable of expressing the human D<sub>4</sub> dopamine receptor in cultures of transformed eukaryotic cells and such cultures of transformed eukaryotic cells that synthesize the human D<sub>4</sub> dopamine receptor.

It is an object of the invention to provide a nucleotide sequence encoding a mammalian dopamine receptor. Further, it is an object of the invention to provide a nucleotide sequence that encodes a mammalian dopamine receptor with novel and distinct pharmacological properties. It is specifically an object of the invention to provide a nucleotide sequence encoding a mammalian dopamine receptor having the particular drug dissociation properties of the human dopamine receptor D<sub>4</sub>. In particular, the mammalian dopamine receptor encoded by the nucleotide sequence of the present invention has a high affinity for the drug clozapine. The human D<sub>4</sub> dopamine receptor embodied in the present invention shows a biochemical inhibition constant (termed K<sub>i</sub>) of 1-40 nanomolar (nM), preferably 1-20 nM, most preferably 11 nM clozapine, as detected by the [<sup>3</sup>H]spiperone binding assay disclosed herein. The human D<sub>4</sub> dopamine receptor embodied in the present invention displays the following pharmacological profile of inhibition of [<sup>3</sup>H]spiperone binding in the [<sup>3</sup>H]spiperone binding assay: spiperone > eticlopride > clozapine > (+)-butaclamol > raclopride > SCH23390. In a preferred embodiment of the invention, the nucleotide sequence encoding a dopamine receptor encodes the human dopamine receptor D<sub>4</sub>. The present invention includes a nucleotide sequence encoding a mammalian dopamine receptor derived from a cDNA molecule isolated from a cDNA library constructed with RNA from the human neuroblastoma cell line SK-N-MC. In this embodiment of the invention, the nucleotide sequence includes 780 nucleotides of the human D<sub>4</sub> dopamine receptor gene comprising transmembrane domains V, VI and VII and 126 nucleotides of 3' untranslated sequence.

The invention also includes a nucleotide sequence derived from human genomic DNA. In this embodiment of the invention, the nucleotide sequence includes 5 kilobases (kb) of human genomic DNA encoding the dopamine receptor D<sub>4</sub>. This embodiment includes the sequences present in the cDNA embodiment, an additional 516 nucleotides of coding sequence comprising transmembrane domains I, II, III, and IV, and 590 nucleotides of 5' untranslated sequence. This embodiment of the invention also contains the sequences of four intervening sequences that interrupt the coding sequence of the human D<sub>4</sub> dopamine receptor gene.

The invention includes a nucleotide sequence of a human D<sub>4</sub> receptor molecule, and includes allelic variations of this nucleotide sequence and the corresponding D<sub>4</sub> receptor molecule, either naturally occurring or the product of in vitro chemical or genetic modification, having essentially the same nucleotide sequence as the nucleotide sequence of the human D<sub>4</sub> receptor disclosed herein, wherein the resulting human D<sub>4</sub> receptor molecule has substantially the same drug dissociation properties of the human D<sub>4</sub> receptor molecule corresponding to the nucleotide sequence described herein.

The invention also includes a predicted amino acid sequence for the human D<sub>4</sub> dopamine receptor deduced from the nucleotide sequence comprising the complete coding sequence of the D<sub>4</sub> dopamine receptor gene.

This invention provides both nucleotide and amino acid probes derived from these sequences. The invention includes probes isolated from either the cDNA or genomic DNA clones, as well as probes made synthetically with the sequence information derived therefrom. The invention specifically includes but is not limited to oligonucleotide, nick-translated, random primed, or in vitro amplified probes made using the cDNA or genomic clone embodying the invention, and oligonucleotide and other synthetic probes synthesized chemically using the nucleotide sequence information of the cDNA or genomic clone embodiments of the invention.

It is a further object of this invention to provide sequences of the human D<sub>4</sub> dopamine receptor for use as a probe to determine the pattern, amount and extent of expression of this receptor in various tissues of mammals, including humans. It is also an object of the present invention to provide probes derived from the sequences of the human D<sub>4</sub> dopamine receptor to be used for the detection and diagnosis of genetic diseases. It is an object of this invention to provide probes derived from the sequences of the human D<sub>4</sub> dopamine receptor to be used for the detection of novel related receptor genes.

The present invention also includes synthetic peptides made using the nucleotide sequence information comprising the cDNA or genomic clone embodiments of the invention. The invention includes either naturally occurring or synthetic peptides which may be used as antigens for the production of D<sub>4</sub> dopamine receptor-specific antibodies, or used for competitors of the D<sub>4</sub> receptor molecule for drug binding, or to be used for the production of inhibitors of the binding of dopamine or dopamine analogs of the D<sub>4</sub> dopamine receptor molecule.

In addition, this invention includes a cloning vector comprising the human D<sub>4</sub> dopamine receptor and sequences that mediate the replication and selected growth of microorganisms that carry this vector.

The present invention provides a recombinant expression vector comprising the nucleotide sequence of the human D<sub>4</sub> dopamine receptor and sequences sufficient to direct the synthesis of human D<sub>4</sub> dopamine receptor in cultures of transformed eukaryotic cells. In a preferred embodiment, the recombinant expression vector is comprised of plasmid sequences derived from the plasmid pCD-PS and a hybrid human D<sub>4</sub> dopamine gene. This hybrid human D<sub>4</sub> dopamine gene is comprised of the entirety of the genomic sequences from a particular D<sub>4</sub> dopamine genomic clone described herein, up to a PstI site located in exon III, followed by the remainder of the coding and 3' untranslated sequences found in a particular human cDNA sequence derived from a human neuroblastoma cell line. This invention includes a



recombinant expression vector comprising essentially the nucleotide sequences of genomic and cDNA clones of the human D<sub>4</sub> dopamine receptor in an embodiment that provides for their expression in cultures of transformed eukaryotic cells.

5 It is also an object of this invention to provide cultures of transformed eukaryotic cells that have been transformed with such a recombinant expression vector and that synthesize human D<sub>4</sub> dopamine receptor protein. In a preferred embodiment, the invention provides monkey COS cells that synthesize human D<sub>4</sub> dopamine receptor protein.

10 The present invention also includes protein preparations of the human D<sub>4</sub> dopamine receptor, and preparations of membranes containing the human D<sub>4</sub> dopamine receptor, derived from cultures of transformed eukaryotic cells. In a preferred embodiment, cell membranes containing human D<sub>4</sub> dopamine receptor protein is isolated from culture of COS-7 cells transformed with a recombinant expression vector that directs the synthesis of human D<sub>4</sub> dopamine receptor.

15 It also an object of this invention to provide the human D<sub>4</sub> dopamine receptor for use in the in vitro screening of novel antipsychotic compounds. In a preferred embodiment, membrane preparations containing the human D<sub>4</sub> dopamine receptor, derived from cultures of transformed eukaryotic cells, are used to determine the drug dissociation properties of antipsychotic compounds in vitro. These properties are then used to characterize novel antipsychotic compounds by comparison to the binding properties of known antipsychotic compounds.

20 The present invention will also be useful for the in vivo detection of dopamine and a dopamine analog, known or unknown, either naturally occurring or as the embodiments of antipsychotic or other drugs.

25 It is an object of the present invention to provide a method for the quantitative detection of dopamine and a dopamine analog, either naturally occurring or as the embodiments of antipsychotic or other drugs. It is an additional object of the invention to provide a method to detect dopamine or a dopamine

analog in blood, saliva, semen, cerebrospinal fluid, plasma, lymph, or any other bodily fluid.

## DESCRIPTION OF THE DRAWINGS

Figure 1. The structure of a genomic clone comprising the human D<sub>4</sub> dopamine receptor gene.

5 Restriction map of the genomic human dopamine D<sub>4</sub> receptor clone and alignment with the genomic intron/exon organization of the human dopamine D<sub>2</sub> receptor. Relevant restriction endonuclease sites in the D<sub>4</sub> receptor are indicated. The SallI site is part of the cloning site in EMBL3. The proposed coding regions are boxed and numbered in Roman numerals. Perfect matches of proposed  
10 intron/exon junction sites are indicated by connecting stippled bars between the receptor clones.

Figure 2. The nucleotide sequence of genomic and cDNA clones of human D<sub>4</sub> dopamine receptor gene.

15 Nucleotide and deduced amino acid sequence of the human dopamine receptor gene and cDNA. The putative coding sequence is in capitals (non-coding sequence is in italics) and deduced amino acid sequence is shown above the nucleotide sequence. Numbering of the putative coding sequence begins with the first methionine of the open reading frame. The sequence corresponding to the  
20 cDNA clone is hatched.

Figure 3. Amino acid sequence alignment of mammalian dopamine receptors

25 Alignment of the putative amino acid sequence of the human D<sub>4</sub> receptor with the human and rat D<sub>2</sub>, rat D<sub>3</sub> and human and rat D<sub>1</sub> receptors. Amino acids conserved within the group of dopamine receptors are shaded. The putative transmembrane domains are overlined and labeled by Roman numerals.

Figure 4. Binding of [<sup>3</sup>H]spiperone to transfected COS-7 cell membranes.

30 Saturation isotherms of the specific binding of [<sup>3</sup>H]spiperone to membranes from COS-7 cells expressing the cloned human dopamine D<sub>4</sub> receptor. The results shown are representative of two independent experiments each conducted in duplicate. Inset, Scatcherd plot of the same data. Estimated B(max) (approximately 260 fmol/mg protein) and K(1) (70 pM) values were  
35 obtained by LIGAND computer program.

Figure 5. Pharmacological specificity of [ $^3$ H]spiperone binding to transfected C6S-7 cells.

5 Representative curves are shown for the concentration dependent inhibition of [ $^3$ H]spiperone binding by various dopaminergic agonist and antagonists. Data were analyzed by LIGAND and the results shown are the means of duplicate determinations. Estimated  $K_i$  values are listed in Table I along with the  $K_i$  values obtained on the human  $D_2$  receptor expressed in GH(4)ZR(7) cells.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "D<sub>1</sub>-dopamine receptor" as used herein refers to proteins substantially homologous to, and having substantially the same biological activity as, the protein coded for by the nucleotide sequence depicted in Figure 2 (i.e., proteins which display high affinity binding to clozapine). This definition is intended to encompass natural allelic variations in the D<sub>1</sub>-dopamine receptor sequence. Cloned genes of the present invention may code for D<sub>1</sub>-dopamine receptors of any species of origin, including, mouse, rat, rabbit, cat, and human, but preferably code for receptors of mammalian, most preferably human, origin.

Nucleotide bases are abbreviated herein as follows:

|            |           |
|------------|-----------|
| A-Adenine  | G-Guanine |
| C-Cytosine | T-Thymine |

Amino acid residues are abbreviated herein to either three letters or a single letter as follows:

|                     |                     |
|---------------------|---------------------|
| Ala;A-Alanine       | Leu;L-Leucine       |
| Arg;R-Arginine      | Lys;K-Lysine        |
| Asn;N-Asparagine    | Met;M-Methionine    |
| Asp;D-Aspartic acid | Phe;F-Phenylalanine |
| Cys;C-Cysteine      | Pro;P-Proline       |
| Gln;Q-Glutamine     | Ser;S-Serine        |
| Glu;E-Glutamic acid | Thr;T-Threonine     |
| Gly;G-Glycine       | Trp;W-Tryptophan    |
| His;H-Histidine     | Tyr;Y-Tyrosine      |
| Ile;I-Isoleucine    | Val;V-Valine        |

The production of proteins such as the D<sub>1</sub>-dopamine receptor from cloned genes by genetic engineering is well known. See, e.g., U.S. Patent No. 4,761,371 to Bell et al. at Col. 6 line 3 to Col. 9 line 65. (The disclosure of all U.S. patent references cited herein is to be incorporated herein by reference.) The discussion which follows is accordingly intended as an overview of this field, and is not intended to reflect the full state of the art.

DNA which encodes the D<sub>4</sub>-dopamine receptor may be obtained, in view of the instant disclosure, by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures, as illustrated below. Screening of mRNA or genomic DNA may be carried out with oligonucleotide probes generated from the D<sub>4</sub>-dopamine receptor gene sequence information provided herein. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with known procedures and used in conventional hybridization assays, as described in greater detail in the Examples below. In the alternative, D<sub>4</sub>-dopamine receptor gene sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the D<sub>4</sub>-dopamine receptor gene sequence provided herein. See U.S. Patent Nos. 4,683,195 to Mullis *et al.* and 4,683,202 to Mullis.

The D<sub>4</sub>-dopamine receptor may be synthesized in host cells transformed with vectors containing DNA encoding the D<sub>4</sub>-dopamine receptor. A vector is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding the D<sub>4</sub>-dopamine receptor and/or to express DNA which encodes the D<sub>4</sub>-dopamine receptor. An expression vector is a replicable DNA construct in which a DNA sequence encoding the D<sub>4</sub> receptor is operably linked to suitable control sequences capable of effecting the expression of the D<sub>4</sub> receptor in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred

by an origin of replication, and a selection gene to facilitate recognition of transformants.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). The vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. Transformed host cells are cells which have been transformed or transfected with the D<sub>4</sub> receptor vectors constructed using recombinant DNA techniques. Transformed host cells ordinarily express the D<sub>4</sub> receptor, but host cells transformed for purposes of cloning or amplifying the D<sub>4</sub> receptor DNA need not express the D<sub>4</sub> receptor. When expressed, the D<sub>4</sub> receptor will typically be located in the host cell membrane.

DNA regions are operably linked when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leaders sequences, contiguous and in the same translational reading frame.

Cultures of cells derived from multicellular organisms are a desirable host for recombinant D<sub>4</sub>-dopamine receptor synthesis. In principal, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. However, mammalian cells are preferred, as illustrated in the Examples. Propagation of such cells in cell culture has become a routine procedure. See Tissue Culture, Academic Press, Kruse and Patterson, editors (1973). Examples of useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI138, BHK, COS-7, CV, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an

origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome binding site, RNA splice site (if intron-containing genomic DNA is used), a polyadenylation site, and a transcriptional termination sequence.

5       The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells are often provided by viral sources. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and Simian Virus 40 (SV40). See, e.g., U.S. patent No. 4,599,308. The  
10       early and late promoters of SV40 are useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. See Fiers *et al.*, Nature 273, 113 (1978). Further, the human genomic D<sub>4</sub> receptor promoter, control and/or signal sequences, may also be used,  
15       provided such control sequences are compatible with the host cell chosen.

      An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral source (e.g., Polyoma,  
20       Adenovirus, VSV, or MPV), or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter may be sufficient.

      D<sub>4</sub>-dopamine receptors made from cloned genes in accordance with the present invention may be used for screening compounds  
25       for D<sub>4</sub> dopamine receptor activity, or for determining the amount of a dopaminergic drug in a solution (e.g., blood plasma or serum). For example, host cells may be transformed with a vector of the present invention, D<sub>4</sub>-dopamine receptors expressed in that host, the cells lysed, and the membranes from those cells used to  
30       screen compounds for D<sub>4</sub>-dopamine receptor binding activity. Competitive binding assays in which such procedures may be carried out are well known, as illustrated by the Examples below. By selection of host cells which do not ordinarily express a dopamine receptor, pure preparations of membranes containing D<sub>4</sub>  
35       receptors can be obtained. Further, D<sub>4</sub>-dopamine receptor agonist and antagonists can be identified by transforming host cells with



vectors of the present invention. Membranes obtained from such cells can be used in binding studies wherein the drug dissociation activity is monitored. Such cells must contain D<sub>4</sub> protein in the plasma and other cell membranes. Procedures for carrying out assays such as these are also described in greater detail in the Examples which follow.

Cloned genes and vectors of the present invention are useful in molecular biology to transform cells which do not ordinarily express the D<sub>4</sub>-dopamine receptor to thereafter express this receptor. Such cells are useful as intermediates for making cell membrane preparations useful for receptor binding assays, which are in turn useful for drug screening. Further, genes and vectors of the present invention are useful in gene therapy. For such purposes, retroviral vectors as described in U.S. Patent No. 4,650,764 to Temin and Watanabe or U.S. Patent No. 4,861,719 to Miller may be employed. Cloned genes of the present invention, or fragments thereof, may also be used in gene therapy carried out homologous recombination or site-directed mutagenesis. See generally Thomas, K. and Capecchi, M., Cell 51, 503-512 (1987); Bertling, W., Bioscience Reports 7, 107-112 (1987); Smithies, O. et al., Nature 317, 230-234 (1985).

Cloned genes of the present invention, and oligonucleotides derived therefrom, are useful for screening for restriction fragment length polymorphism (RFLP) associated with certain genetic disorders.

Oligonucleotides of the present invention are useful as diagnostic tools for probing D<sub>4</sub>-receptor gene expression in nervous tissue. For example, tissue can be probed in situ with oligonucleotide probes carrying detectable groups by conventional autoradiography techniques, as explained in greater detail in the Examples below, to investigate native expression of this receptor or pathological conditions relating thereto. Further, chromosomes can be probed to investigate the presence or absence of a D<sub>4</sub>-dopamine receptor gene, and potential pathological conditions related thereto, as also illustrated by the Examples below.

The Examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

## EXAMPLE 1

Screening Tissue and Cell Line RNA for  
Dopamine-Like Receptor Expression

RNA was prepared from different rat tissues or cell lines  
5 using the guanidium thiocyanate/CsCl procedure described in  
Bunzow et al., Nature 336: 783-787 (1988). The tissues included  
heart, epididymis, testis, gut, pancreas, spleen, thymus, muscle,  
ventricle, atria, lung, adrenal, kidney, liver, pineal gland and  
pituitary. The cell lines screened included SK-N-MC, SK-N-SH,  
10 COS, AKR1, Ltk<sup>-</sup>, GH4C1, NG108-15, AtT20, 3T3, BSC40, C6, CV-1,  
Hela, IMR-32, N4TG1, NCB-20, PC-12, Rion m5f and WERI-Rb-1. 20  
 $\mu$ g of RNA was analyzed by Northern blot hybridization with a  
radiolabeled BstYI-BglII DNA fragment of the rat D<sub>2</sub> receptor,  
which encodes the putative transmembrane domains VI and VII. The  
15 hybridization conditions used were 25% formamide, 1M NaCl, 1%  
SDS, 100  $\mu$ g/ml denatured salmon sperm DNA, 0.2%  
polyvinylpyrrolidone, 0.2% Ficoll, and 0.05M Tris/HCl (pH 7.4);  
hybridization was performed overnight at 37°C. The blot was then  
washed at 55°C in 2X standard saline-citrate (SSC) and 1% sodium  
20 dodecyl sulfate (SDS). Exposure was for two days at -70°C using  
an intensifying screen. For comparison, the same blot was  
hybridized under high stringency conditions, which are the same  
conditions using 50% formamide and 42°C for the hybridization and  
0.2X SSC for the wash. Under high and low stringency only the  
25 adrenal gland showed a positive signal while under low stringency  
the SK-N-MC line also showed a signal.

## EXAMPLE 2

Construction of a cDNA Phage Library using Neuroblastoma RNA

Double-stranded cDNA was synthesized using standard techniques [see Sambrook, J. et al. Molecular Cloning: A Laboratory Manual 2d ed. Cold Spring Harbor Laboratory Press 1989] from poly(A)+ mRNA isolated from the human neuroblastoma cell line SK-N-MC as described in Example 1. The cDNA was directionally cloned into the EcoRI and XhoI restriction endonuclease sites of the phage cloning vector lambda ZAPII (Stratagene, 11099 North Torrey Pines Road, La Jolla, CA 92037). The library was transferred to colony plaque screen filters (New England Nuclear, 549 Albany Street, Boston, MA 02118) and prehybridized overnight at 37°C in 25% formamide, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.05 M Tris/HCl (pH 7.5), 1 M NaCl, 0.1% pyrophosphate, 1% SDS and denatured salmon sperm DNA (100 µg/ml). Approximately 500,000 independent clones were screened under low-stringency conditions of hybridization. Hybridization was performed for 30 hrs with 1.6 kb BamHI - BglII and 300 bp BstYI - BglII fragments of a rat D<sub>2</sub> receptor clone, <sup>32</sup>P-labeled using a random primed labeling system (Boehringer Mannheim Biochemicals, P.O. Box 50414, Indianapolis, IN 46250) at a specific activity of 10<sup>8</sup> dpm/µg. Filters were washed at 55°C in 2X SSC and 1% SDS. The clone D<sub>2</sub>210S was isolated and sequenced using the Sanger dideoxy chain termination method catalyzed by Sequenase (U.S. Biochemical Corporation, P. O. Box 22400, Cleveland, OH 44122). The sequence of this clone is shown in Figure 2 (hatched area).

**EXAMPLE 3****Screening a Genomic DNA Phage Library  
with a Human Dopamine Receptor Probe**

5        Clone D<sub>2</sub>10S was <sup>32</sup>P-labeled by random primed synthesis and  
used to screen a commercially available human genomic library  
cloned in the phage vector EMBL3 (Clontech). Hybridization was  
performed as described in Example 2 using with 50% formamide.  
After hybridization the filters were washed at 65°C in 0.1X SSC  
and 0.1% SDS. The clone D<sub>2</sub>10G was isolated and analyzed by  
10    restriction endonuclease and Southern blot analysis. The map of  
this genomic clone is shown in Figure 1, wherein the structure of  
the D<sub>4</sub> receptor gene is compared with the structure of the D<sub>2</sub>  
gene. 1.3 kb and 2.6 kb PstI - PstI fragments and an overlapping  
2.0 kb SalI - EcoRI fragment of the D<sub>4</sub> receptor gene were  
15    subcloned into the plasmid pBluescript-SK (Stratagene). The  
subcloned fragments were characterized by sequence analysis as  
described above. This sequence is shown in Figure 2.

## EXAMPLE 4

DNA Sequence Analysis of the Human D<sub>2</sub> Dopamine Receptor

One of the cDNA clones detected by screening the SK-N-MC neuroblastoma library with a rat D<sub>2</sub> probe at low stringency (D<sub>2</sub>10S) contained a 780 bp EcoRI-XhoI insert which hybridized to the rat probe. Sequence analysis of this insert showed the presence of an open reading frame with homology to the amino acid sequence of transmembrane domains V (45%), VI (46%) and VII (78%) of the D<sub>2</sub> receptor as shown in Figure 3.

Screening of a human genomic EMBL3 library (Clontech) under high stringency conditions with the clone D<sub>2</sub>10S resulted in the isolation of the genomic clone D<sub>2</sub>10G. Southern blot and sequence analysis indicated that the clone contained a 5 kb SalI-PstI fragment which coded for the entire gene of D<sub>2</sub>10S. Sequence analysis revealed, 590 bp downstream from the SalI site, a potential translation initiation codon (ATG) followed by an open reading frame that showed amino acid sequence homology with transmembrane domain I (36%) and II (63%) of the D<sub>2</sub> receptor. Almost immediately downstream from transmembrane domain II, homology to the D<sub>2</sub> receptor disappears, indicating the presence of an intron in the genomic DNA. This intron spanned approximately 2 kb, after which sequence homology to the D<sub>2</sub> receptor was re-established. Translation of the putative gene product showed homology to the transmembrane domains III (68%), IV (37%), V(46%) and VII (78%) of the D<sub>2</sub> receptor (see Figure 3).

Potential splice junction donor and acceptor sites (Mount Nucl. Acids Res. 10: 461-472, 1982) were found in the transmembrane domains II, III and VI, as shown in Figure 1. These splice sites were at an identical position as in the D<sub>2</sub> and D<sub>3</sub> receptor gene [see Grandy, D.K. et al. Proc. Natl. Acad. Sci. U.S.A. 86: 9762-9766 (1989); Dal Toso, R. et al. EMBO J. 8: 4025-4034 (1989); Sokoloff, P. et al. Nature 347: 146-151 (1990)] and Figure 1. The coding sequence downstream from transmembrane domain IV is identical to the sequence of clone D<sub>2</sub>10S but is interrupted by an intron of about 300 bp between transmembrane domain V and VI and an additional intron of 92 bp in

transmembrane VI (Figure 1, hatched area). The precise location of the splice site for the intron between transmembrane V and VI cannot be determined due to the fact that a sequence of 52 bp present in the coding sequence is repeated exactly on either side of the intron (Figure 2).

The deduced amino acid sequence from the genomic and cDNA nucleotide sequences indicated that this gene codes for a protein of 387 amino acids with an apparent molecular weight of 41kD. A hydrophobicity plot of the protein sequence suggests the existence of seven transmembrane domains. These regions correlate with the observed homologous regions in the human D<sub>2</sub> receptor and other receptors belonging to the family of G-protein coupled receptors [Bunzow, J.R. *et al.* Nature 336: 783-787 (1988); Sokoloff, P. *et al.* Nature 347: 146-151 (1990); Dohlman, H.G. *et al.* Biochemistry 26: 2657-2664 (1987) and Figure 2]. Two amino acids downstream from the initiation methionine is a potential N-linked glycosylation site [Hubbard, S. & Ivatt, R. Annu. Rev. Biochem. 50: 555-583 (1981)]. The amino acid residues Asp (80) and Asp (115) in the D<sub>4</sub> receptor, which are conserved within the family catecholaminergic receptors, are postulated to act as centurions in catecholamine binding [Strader, C.D. *et al.* J. Biol. Chem. 263: 10267-10271 (1988)]. Also conserved within the family of catecholaminergic receptors are Ser (197) and Ser (700) which have been suggested to interact with the catechol hydroxyl groups [Kozak, M. Nucleic Acids Res. 12: 857-872 (1984)]. Several consensus sites for potential phosphorylation by protein kinase C and protein kinase A are noted in the 3rd cytoplasmic loop [Sibley, D.R. *et al.* Cell 48: 913-922 (1987); Bouvier, M. *et al.* Nature 333: 370-373 (1988)]. The Cys (187), which may serve as a substrate for palmitoylation, is conserved in most of the G-protein coupled receptors [O'Dowd, B.F. *et al.* J. Biol. Chem 264: 7564-7569 (1989)]. The short carboxyl tail, which terminates similar to the D<sub>2</sub> and D<sub>3</sub> receptor at Cys (387) [Bunzow, J.R. *et al.* Nature 336: 783-787 (1988); Grandy, D.K. *et al.* Proc. Natl. Acad. Sci. U.S.A. 86: 9762-9766 (1989); Dal Toso, R. *et al.* EMBO J. 8: 4025-4034 (1989);

Sokoloff, P. et al. Nature 347: 146-151 (1990)], and the relative large 3rd cytoplasmic loop, are features observed in most receptors which interact with an isoform of the G protein.



## EXAMPLE 5

Construction of an Mammalian DNA Expression  
Vector using Dopamine Receptor cDNA

5 The SalI-PstI gene fragment (Figure 1, the PstI site found  
in exon III after transmembrane domain V) was ligated to the  
corresponding PstI-EcoRI cDNA fragment isolated from the SK-N-MC  
cDNA. This construct was then cloned into the vector pCD-PS  
[Bonner et al. Neuron 1: 403-410 (1988)]. This vector allows for  
the expression of the human D<sub>1</sub> receptor gene from the SV40  
10 promoter. Large quantities of the pCD-PS-D<sub>1</sub> construct plasmid  
were prepared using standard techniques. This plasmid was  
transfected into COS-7 cells by the calcium phosphate  
precipitation technique [Gorman et al. Science 221: 551-553  
(1983)]. Two days later membranes cells were harvested and  
15 analyzed as described in Example 6.

## EXAMPLE 6

Analysis of Dopamine and Dopamine-Antagonist  
Binding of D<sub>1</sub> Dopamine Receptor

Cells were harvested and homogenized using a teflon pestle  
5 in 50 mM Tris-HCl (pH 7.4 at 4°C) buffer containing 5 mM EDTA,  
1.5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 5 mM KCl and 120 mM NaCl. Homogenates  
were centrifuged for 15 minutes at 39,000 g, and the resulting  
pellets resuspended in buffer at a concentration of 150-250  
10 ug/ml. For saturation experiments, 0.25 ml aliquots of tissue  
homogenate were incubated in duplicate with increasing  
concentrations of [<sup>3</sup>H]spiperone (70.3 Ci/mmol; 10-3000 pM final  
concentration) for 120 min at 22°C in a total volume of 1 ml.  
The results of these experiments are shown in Figure 4. For  
15 competition binding experiments, assays were initiated by the  
addition of 0.25 ml of membrane and incubated in duplicate with  
the concentrations of competing ligands indicated in Figure 5  
(10<sup>-14</sup> to 10<sup>-3</sup> M) and [<sup>3</sup>H]spiperone (150-300 pM) for 120 min at  
22°C. Assays were terminated by rapid filtration through a  
20 Titertek cell harvester and filters subsequently monitored to  
quantitate radioactive tritium. For all experiments specific  
[<sup>3</sup>H]spiperone binding was defined as that inhibited by 10 μM  
(+)-sulpiride. Both saturation and competition binding data were  
analyzed by the non-linear least square curve-fitting program  
25 LIGAND run on a Digital Micro-PP-11. The human D<sub>1</sub> dopamine  
receptor displays the following pharmacological profile of  
inhibition of [<sup>3</sup>H]spiperone binding in this assay: spiperone >  
eticlopride > clozapine > (+)-butaclamol > raclopride > SCH23390.

## WHAT WE CLAIM IS:

1. A DNA sequence comprising a nucleotide sequence encoding a mammalian dopamine receptor, wherein the mammalian dopamine receptor has the drug dissociation properties of the human dopamine receptor D<sub>4</sub>.

2. A DNA sequence according to Claim 1 wherein the mammalian dopamine receptor encoded is the human D<sub>4</sub> dopamine receptor.

3. A DNA sequence according to Claim 1 wherein the mammalian dopamine receptor encoded therein has the drug dissociation properties described in Table 1.

4. A DNA sequence according to Claim 1 wherein the mammalian dopamine receptor encoded therein has a high affinity for the drug clozapine.

5. A homogeneous composition of a 41 kilodalton human dopamine receptor D<sub>4</sub> or derivative thereof, wherein the amino acid sequence of the dopamine receptor or derivative thereof comprises a sequence shown in Figure 1.

6. A probe for the detection of human D<sub>4</sub> dopamine receptor expression comprising the nucleotide sequence of Claim 2.

7. A probe according to Claim 6 whereby the probe is adapted for use in the detection and diagnosis of genetic disease in a human.

8. A probe as in Claim 6 whereby the probe is adapted for use in the detection, isolation and characterization of novel related mammalian receptor genes.

9. A cloning vector comprising a nucleotide sequence encoding the human dopamine receptor D<sub>4</sub>.

10. A recombinant expression vector comprising the DNA sequence of Claim 2, wherein the vector is capable of expressing the human dopamine receptor D<sub>4</sub> in a transformed eukaryotic cell culture.

11. A eukaryotic cell culture transformed with the expression vector of Claim 10, wherein the transformed eukaryotic

cell culture is capable of expressing the human dopamine receptor D<sub>4</sub>.

12. A method of screening a compound as an inhibitor of dopamine binding to the human dopamine receptor D<sub>4</sub>, the method comprising the following steps:

(a) transforming a eukaryotic cell culture with an expression vector as in Claim 10 capable of expressing the human dopamine receptor D<sub>4</sub> in a eukaryotic cell; and

(b) assaying for ability of the compound to inhibit the binding of a detectable dopamine analog.

13. A method of screening a compound for anti-psychotic activity, the method comprising the following steps:

(a) transforming a eukaryotic cell culture with an expression vector as in Claim 10 capable of expressing the human dopamine receptor D<sub>4</sub> in a eukaryotic cell;

(b) assaying for ability of the compound to inhibit the binding of a detectable dopamine analog; and

(c) testing those drugs for anti-psychotic activity based on their affinity for the D<sub>4</sub> dopamine receptor.

14. A method of quantitatively detecting a compound as an inhibitor of dopamine binding to the human dopamine receptor D<sub>4</sub>, the method comprising the following steps:

(a) transforming a eukaryotic cell culture with an expression vector as in Claim 10 capable of expressing the human dopamine receptor D<sub>4</sub> in a eukaryotic cell; and

(b) assaying for amount of a compound by measuring the extent of inhibition of binding of a detectable dopamine analog.

15. The method of Claim 14 wherein the compound to be tested is present in a human.

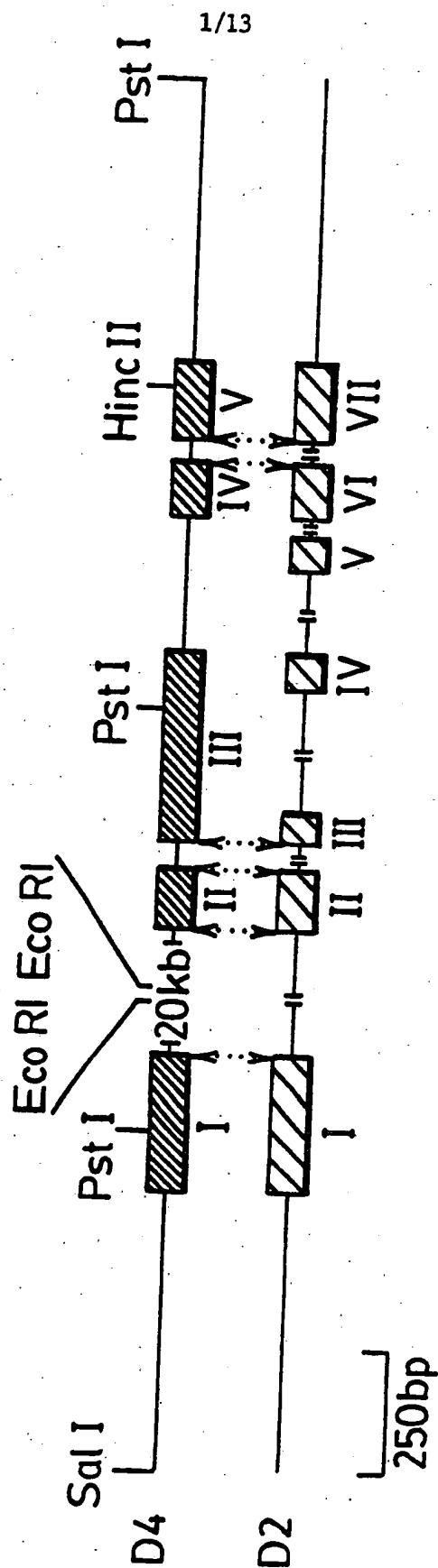
16. The method of Claim 14 wherein the compound is present in human blood.

17. The method of Claim 14 wherein the compound is present in human cerebrospinal fluid.

18. The method of Claim 14 wherein the compound is present in human brain.

19. The method of Claim 14 wherein the compound is unknown.

FIG. 1



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FIG. 2

5' -CGGGGGCGGGACCAGGGTCCGGCCGGGGCGTGCCCCC  
 GGGGAGGGACTCCCCGGCTTGCCCCCGGCGTTGTCCGCGGTG  
 CTCAGCGCCCGCCCGGGCGCGCC<sup>+1</sup> ATG GGG AAC CGC AGC  
 MET GLY ASN ARG SER  
 48  
 ACC GCG GAC GCG GAC GGG CTG CTG GCT GGG CGC  
 THR ALA ASP ALA ASP GLY LEU LEU ALA GLY ARG  
 GGG CGG GCC GCG GGG GCA TCT GCG GGG GCA TCT  
 GLY PRO ALA ALA GLY ALA SER ALA GLY ALA SER  
 114  
 GCG GGG CTG GCT GGG CAG GGC GCG GCG GCG CTG  
 ALA GLY LEU ALA GLY GLN GLY ALA ALA ALA LEU  
 GTG GGG GGC GTG CTG CTC ATC GGC GCG GTG CTC  
 VAL GLY GLY VAL LEU LEU ILE GLY ALA VAL LEU  
 180  
 GCG GGG AAC TCG CTC GTG TGC GTG AGC GTG GCC  
 ALA GLY ASN SER LEU VAL CYS VAL SER VAL ALA  
 ACC GAG CGC GCC CTG CAG ACG CCC ACC AAC TCC  
 THR GLU ARG ALA LEU GLN THR PRO THR ASN SER  
 246  
 TTC ATC GTG AGC CTG GCG GCC GCC GAC CTC CTC  
 PHE ILE VAL SER LEU ALA ALA ALA ASP LEU LEU  
 CTC GCT CTC CTG GTG CTG CCG CTC TTC GTC TAC  
 LEU ALA LEU LEU VAL LEU PRO LEU PHE VAL TYR  
 TCC GAG GTGAGCCGCGTCCGGCCGCA.....  
 SER GLU  
 ...CCTGTGGTGTGCGCCGCGCAG GTC CAG GGT GGC GCG  
 VAL GLN GLY GLY ALA  
 333  
 TGG CTG CTG AGC CCC CGC CTG TGC GAC GCC CTC  
 TRP LEU LEU SER PRO ARG LEU CYS ASP ALA LEU

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FIG. 2 cont.

ATG GCC ATG GAC GTC ATG CTG TGC ACC GCC TCC  
 MET ALA MET ASP VAL MET LEU CYS THR ALA SER  
 398

ATC TTC AAC CTG TGC GCC ATC AGC GTG GAC AG  
 ILE PHE ASN LEU CYS ALA ILE SER VAL ASP ARG

GTGCGCCGCCCTCCCCGCCCGCGCCCCGGCGCCCCCGCGCCCC

GCCCGCCGCCCTCACCGCGGCCTGTGCGCTGTCCGGCGCCCC

TCGGCGCTCCCCGCAG G TTC GTG GCC GTG GCC GTG  
 PHE VAL ALA VAL ALA VAL  
 450

CCG CTG CGC TAC AAC CGG CAG GGT GGG AGC CGC  
 PRO LEU ARG TYR ASN ARG GLN GLY GLY SER ARG

CGG CAG CTG CTG CTC ARC GGC GCC ACG TGG CTG  
 ARG GLN LEU LEU LEU ILE GLY ALA THR TRP LEU  
 516

CTG TCC GCG GCG GTG GCG GCG CCC GTA CTG TGC  
 LEU SER ALA ALA VAL ALA ALA PRO VAL LEU CYS

~~GGC CTC AAC GAC GTG CGC GGC CGC GAC CCC GCC~~  
~~GLY LEU ASN ASP VAL ARG GLY ARG ASP PRO ALA~~  
 582

~~GTG TGC CGC CTG GAG GAC CGC GAC TAC GTG GTC~~  
~~VAL CYS ARG LEU GLU ASP ARG ASP TYR VAL VAL~~

~~TAC TCG TCC GTG TGC TCC TTC TTC CTA CCC TGC~~  
~~TYR SER SER VAL CYS SER PHE PHE LEU PRO CYS~~  
 648

~~CCG CTC ATG CTG CTG CTG TAC TGG GCC ACG TTC~~  
~~PRO LEU MET LEU LEU LEU TYR TRP ALA THR PHE~~

~~CGC GGC CTG CAG CGC TGG GAG GTG GCA CGT CGC~~  
~~ARG GLY LEU GLN ARG TRP GLU VAL ALA ARG ARG~~  
 714

~~GCC AAG CTG CAC GGC CGC GCG CCC CGC CGA CCC~~  
~~ALA LYS LEU HIS GLY ARG ALA PRO ARG ARG PRO~~



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FIG. 2 cont.

|     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| AGC | GGC | CCT | GGC | CCG | CCT | TCC | CCC | ACG | CCA | CCC |
| SER | GLY | PRO | GLY | PRO | PRO | SER | PRO | THR | PRO | PRO |

780

|     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| GCG | CCC | CGC | CTC | CCC | CAG | GAC | CCC | TGC | GGC | CCC |
| ALA | PRO | ARG | LEU | PRO | GLN | ASP | PRO | CYS | GLY | PRO |

■

|     |     |     |     |     |     |     |     |     |          |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|----------|
| GAC | TGT | GCG | CCC | CCC | GCG | CCC | GGC | CT  | TCCCCGGG |
| ASP | CYS | ALA | PRO | PRO | ALA | PRO | GLY | LEU |          |

GTCCCTGCGGCC.....CCTGTGCGCCCCCGCGCCCGGCCT

CCCCCAGGACCCCTGCGGCCCCGACTGTGCGCCCCCGCGCCC

834

|       |     |     |     |     |     |     |     |     |     |     |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| GGCCT | C   | CCC | CCG | GAC | CCC | TGC | GGC | TCC | AAC | TGT |
|       | PRO | PRO | ASP | PRO | CYS | GLY | SER | ASN | CYS |     |

|     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| GCT | CCC | CCC | GAC | GCC | GTC | AGA | GCC | GCC | GCG | CTC |
| ALA | PRO | PRO | ASP | ALA | VAL | ARG | ALA | ALA | ALA | LEU |

900

|     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| CCA | CCC | CAG | ACT | CCA | CCG | CAG | ACC | CGC | AGG | AGG |
| PRO | PRO | GLN | THR | PRO | PRO | GLN | THR | ARG | ARG | ARG |

|     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| CGG | CGT | GCC | AAG | ATC | ACC | GGC | CGG | GAG | CGC | AAG |
| ARG | ARG | ALA | LYS | ILE | THR | GLY | ARG | GLU | ARG | LYS |

□

|     |     |     |     |     |     |     |     |     |   |      |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|------|
| GCC | ATG | AGG | GTC | CTG | CCG | GTC | GTC | GTC | G | GTGG |
| ALA | MET | ARG | VAL | LEU | PRO | VAL | VAL | VAL |   |      |

□

GTTCTGTCTGAGGGGCGGGGAGGAGAGGGGGGGAGTAC

GAGGCCGGCTGGGCGGGGGGCGCTAACGCGGCTCTCGGCGCCC

|      |     |     |     |     |     |     |     |     |     |     |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| CCAG | GG  | GCC | TTC | CTG | CTG | TGC | TGG | ACG | CCC | TTC |
|      | GLY | ALA | PHE | LEU | LEU | CYS | TRP | THR | PRO | PHE |

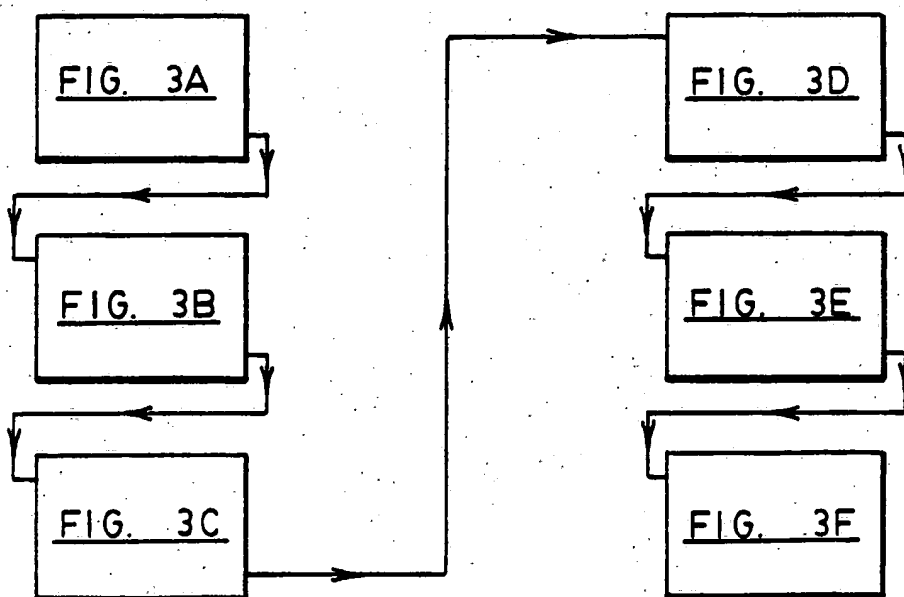
1023

|     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| TTC | GTC | GTC | CAC | ATC | ACG | CAG | GCG | CTG | TGT | CCT |
| PHE | VAL | VAL | HIS | ILE | THR | GLN | ALA | LEU | CYS | PRO |

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FIG. 2 cont.

|                                             |     |      |                               |     |     |     |     |     |     |     |      |
|---------------------------------------------|-----|------|-------------------------------|-----|-----|-----|-----|-----|-----|-----|------|
| GCC                                         | TGC | TCC  | GTG                           | CCC | CCG | CGG | CTG | GTC | AGC | GCC |      |
| ALA                                         | CYS | SER  | VAL                           | PRO | PRO | ARG | LEU | VAL | SER | ALA | 1089 |
| GTC                                         | ACC | TGG  | CTG                           | GGC | TAC | GTC | AAC | AGC | GCC | CTC |      |
| VAL                                         | THR | TRP  | LEU                           | GLY | TYR | VAL | ASN | SER | ALA | LEU |      |
| ACC                                         | CCC | GTC  | ATC                           | TAC | ACT | GTC | TTC | AAC | GCC | GAG |      |
| ASN                                         | PRO | VAL  | ILE                           | TYR | THR | VAL | PHE | ASN | ALA | GLU | 1155 |
| TTC                                         | CGC | AAC  | GTC                           | TTC | CGC | AAG | GCC | CTG | CGT | GCC |      |
| PHE                                         | ARG | ASN  | VAL                           | PHE | ARG | LYS | ALA | LEU | ARG | ALA |      |
| TGC                                         | TGC | TGA  | GCCGGGCACCCCCGGACGCCCCCGGCCTG |     |     |     |     |     |     |     | 1164 |
| CYS                                         | CYS | STOP |                               |     |     |     |     |     |     |     |      |
| ATGGCCAGGCCTCAGGGACCAAGGAGATGGGGAGGGCGCTTTT |     |      |                               |     |     |     |     |     |     |     |      |
| GTACGTTAATTAAACAAATTCCTTCCCAAACTCAGCTGTGAAG |     |      |                               |     |     |     |     |     |     |     |      |
| AAAAAAAAAAAAAAAAAAAA                        |     |      |                               |     |     |     |     |     |     |     |      |
| GCTCCTGGG-3'                                |     |      |                               |     |     |     |     |     |     |     |      |
| AA                                          |     |      |                               |     |     |     |     |     |     |     |      |

FIG. 3

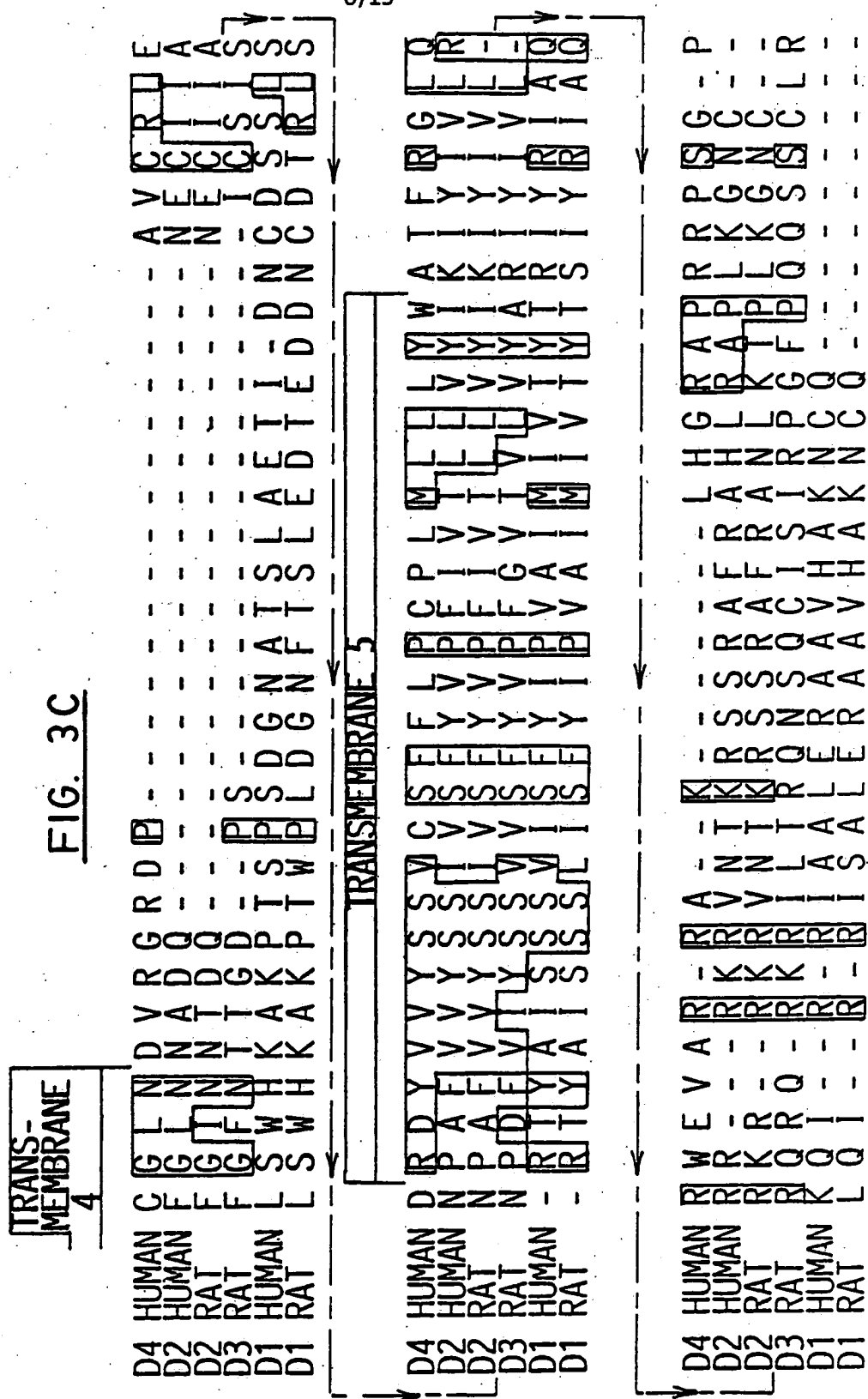
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**FIG. 3C**



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FIG. 3D

|          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D4 HUMAN | G | P | S | T | P | P | A | P | C | R | A | R | L | P | Q | D | S | S | D | P | C | N | A | I | G | P | S | S | G | D | F | F | M | C | A | V | N | H | I | P | R | R | R | D | G | V | M | K | L | D | A | A | P | P | R | R | P |
| D2 HUMAN | T | H | - | D | M | K | L | K | L | L | L | L | V | I | M | F | L | S | S | D | S | N | A | I | G | P | S | S | G | D | F | F | M | C | A | V | N | H | I | P | R | R | R | D | G | V | M | K | L | D | A | A | P | P | R | R | P |
| D2 RAT   | T | H | - | D | M | K | L | K | L | L | L | L | V | I | M | F | L | S | S | D | S | N | A | I | G | P | S | S | G | D | F | F | M | C | A | V | N | H | I | P | R | R | R | D | G | V | M | K | L | D | A | A | P | P | R | R | P |
| D3 RAT   | T | H | - | D | M | K | L | K | L | L | L | L | V | I | M | F | L | S | S | D | S | N | A | I | G | P | S | S | G | D | F | F | M | C | A | V | N | H | I | P | R | R | R | D | G | V | M | K | L | D | A | A | P | P | R | R | P |
| D1 HUMAN | T | H | - | D | M | K | L | K | L | L | L | L | V | I | M | F | L | S | S | D | S | N | A | I | G | P | S | S | G | D | F | F | M | C | A | V | N | H | I | P | R | R | R | D | G | V | M | K | L | D | A | A | P | P | R | R | P |
| D1 RAT   | T | H | - | D | M | K | L | K | L | L | L | L | V | I | M | F | L | S | S | D | S | N | A | I | G | P | S | S | G | D | F | F | M | C | A | V | N | H | I | P | R | R | R | D | G | V | M | K | L | D | A | A | P | P | R | R | P |
| D4 HUMAN | C | G | S | A | M | E | L | L | P | S | S | P | P | T | T | I | Q | R | R | H | P | P | I | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D2 HUMAN | C | G | S | A | M | E | L | L | P | S | S | P | P | T | T | I | Q | R | R | H | P | P | I | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D2 RAT   | C | G | S | A | M | E | L | L | P | S | S | P | P | T | T | I | Q | R | R | H | P | P | I | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D3 RAT   | C | G | S | A | M | E | L | L | P | S | S | P | P | T | T | I | Q | R | R | H | P | P | I | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D1 HUMAN | C | G | S | A | M | E | L | L | P | S | S | P | P | T | T | I | Q | R | R | H | P | P | I | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D1 RAT   | C | G | S | A | M | E | L | L | P | S | S | P | P | T | T | I | Q | R | R | H | P | P | I | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D4 HUMAN | P | D | P | S | H | H | P | S | L | S | S | G | A | T | P | P | G | M | S | S | S | A | E | V | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D2 HUMAN | P | D | P | S | H | H | P | S | L | S | S | G | A | T | P | P | G | M | S | S | S | A | E | V | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D2 RAT   | P | D | P | S | H | H | P | S | L | S | S | G | A | T | P | P | G | M | S | S | S | A | E | V | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D3 RAT   | P | D | P | S | H | H | P | S | L | S | S | G | A | T | P | P | G | M | S | S | S | A | E | V | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D1 HUMAN | P | D | P | S | H | H | P | S | L | S | S | G | A | T | P | P | G | M | S | S | S | A | E | V | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D1 RAT   | P | D | P | S | H | H | P | S | L | S | S | G | A | T | P | P | G | M | S | S | S | A | E | V | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| D4 HUMAN | K | I | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| D2 HUMAN | K | I | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| D2 RAT   | K | I | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| D3 RAT   | K | I | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| D1 HUMAN | K | I | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| D1 RAT   | K | I | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |



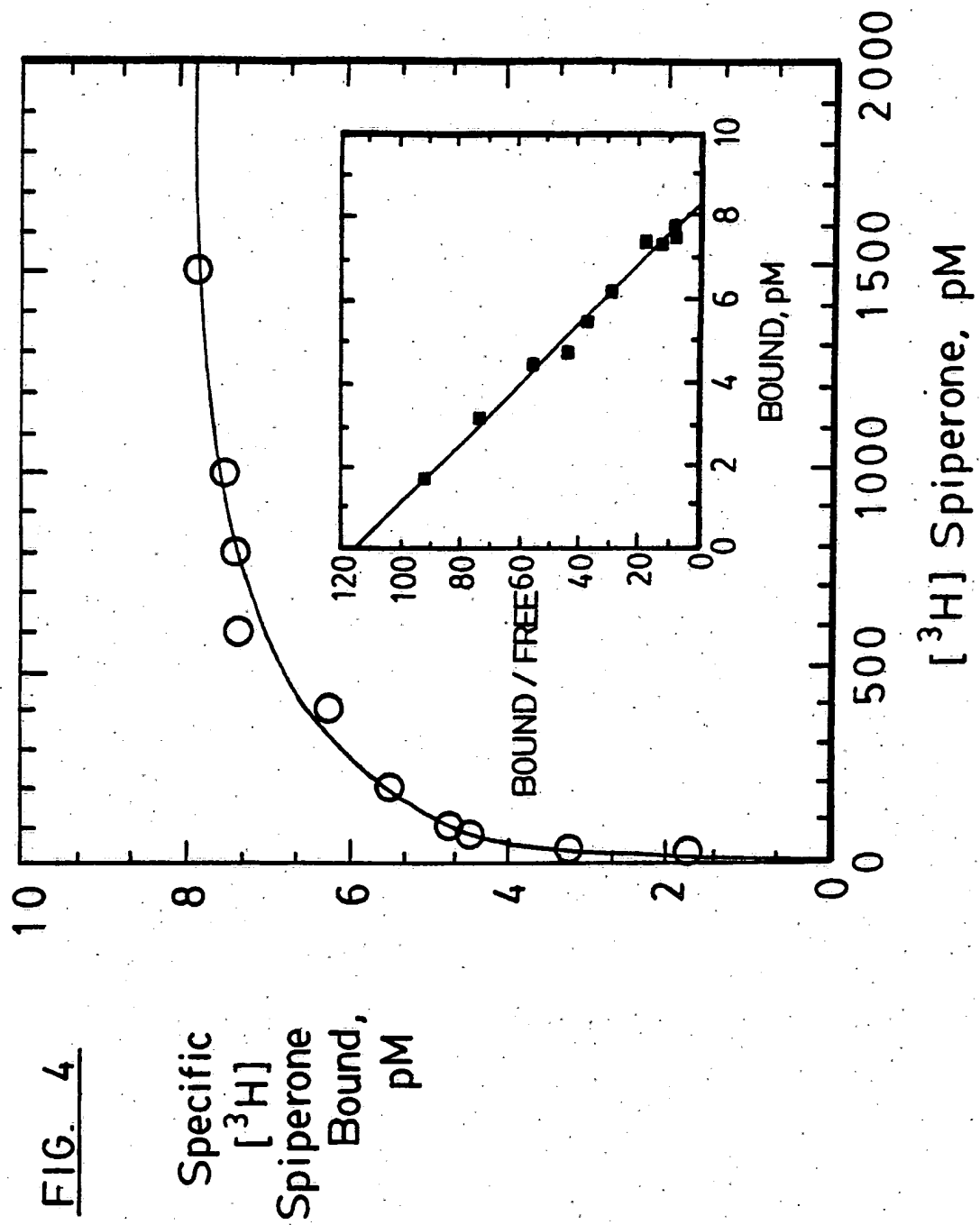
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FIG. 3F

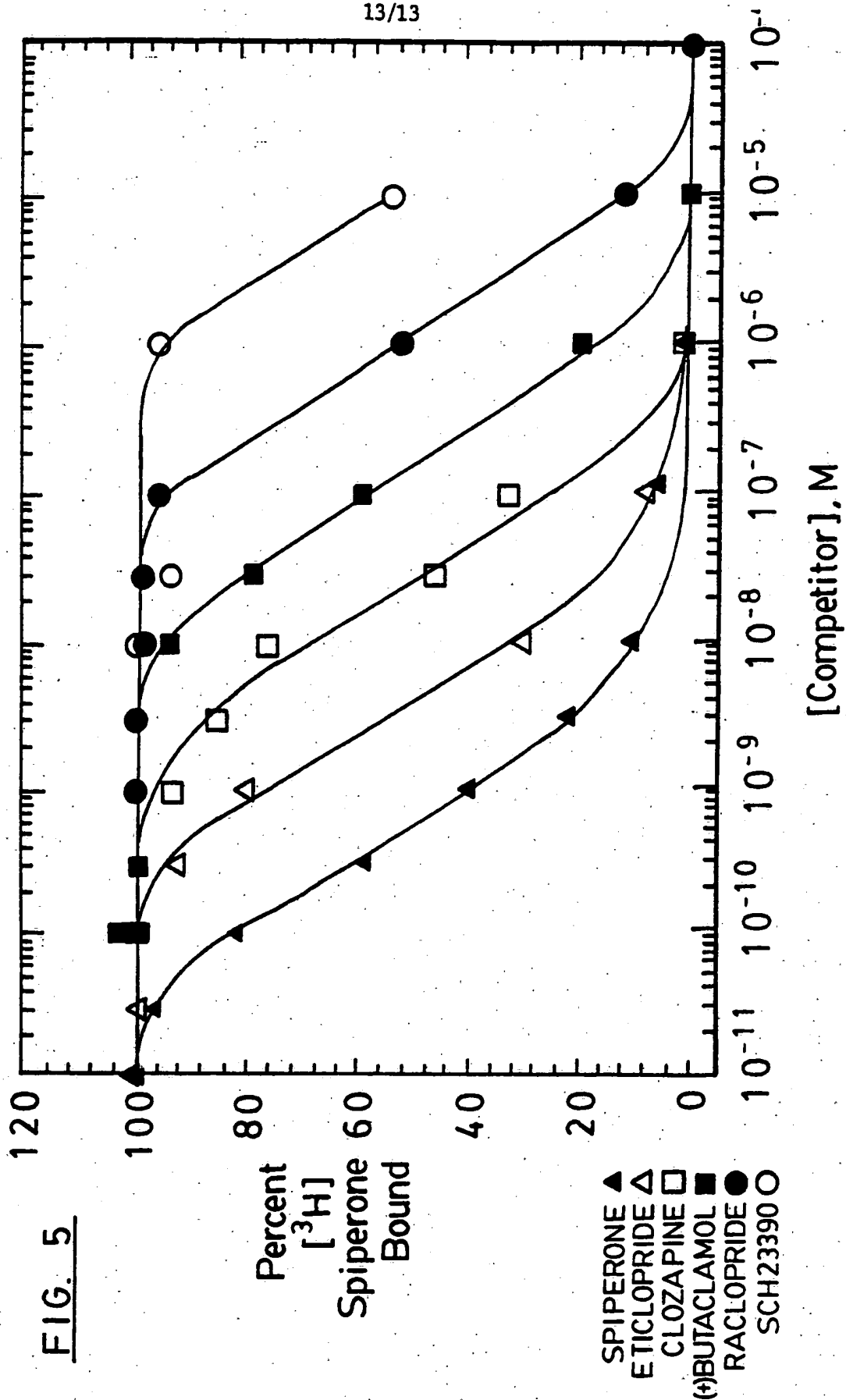
|          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D1 HUMAN | P | A | T | N | N | A | I | E | T | V | S | I | N | N | N | G | A | M | F | S | S | H | H | E | P | R | G | S | I | S | K |
| D1 RAT   | P | T | T | N | N | A | I | E | T | V | S | I | N | N | N | G | A | V | F | S | S | H | H | E | P | R | G | S | I | S | K |
| D1 HUMAN | E | C | N | L | V | Y | L | I | P | H | A | V | G | S | S | E | D | L | K | E | E | A | A | G | I | A | R | P | L | E | K |
| D1 RAT   | D | C | N | L | V | Y | L | I | P | H | A | V | G | S | S | E | D | L | K | E | E | A | A | G | I | A | R | P | L | E | K |
| D1 HUMAN | L | S | P | A | L | S | V | I | L | D | Y | D | T | D | V | S | L | E | K | I | Q | P | I | T | Q | N | G | Q | H | P | T |
| D1 RAT   | L | S | P | A | L | S | V | I | L | D | Y | D | T | D | V | S | L | E | K | I | Q | P | I | T | H | S | G | Q | H | S | T |
| D1 HUMAN |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| D1 RAT   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |



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## INTERNATIONAL SEARCH REPORT

PCT/US 91/09308

International Application No

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|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                        |                                     |
| According to International Patent Classification (IPC) or to both National Classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                        |                                     |
| Int.Cl. 5 C12N15/12;<br>C12N5/10                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | C07K13/00;                                                                                                                                                                                                                                                                             | G01N33/555; G01N33/68               |
| <b>II. FIELDS SEARCHED</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                                                                                                                                                                                        |                                     |
| Minimum Documentation Searched <sup>7</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                        |                                     |
| Classification System                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Classification Symbols                                                                                                                                                                                                                                                                 |                                     |
| Int.Cl. 5                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | C07K ; G01N                                                                                                                                                                                                                                                                            |                                     |
| Documentation Searched other than Minimum Documentation<br>to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                                                        |                                     |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                        |                                     |
| Category <sup>10</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>                                                                                                                                                                         | Relevant to Claim No. <sup>13</sup> |
| P,X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | NATURE.<br>vol. 350, 18 April 1991, LONDON GB<br>pages 160 - 164;<br>Van Tol HH; Bunzow JR; Guan HC; Sunahara RK; Seeman P; Niznik HB; Civelli O: 'Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine.'<br>see the whole document | 1-19                                |
| A                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | NATURE.<br>vol. 347, 13 September 1990, LONDON GB<br>pages 146 - 151;<br>Sokoloff, P. et al: 'Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics.'<br>cited in the application<br>see the whole document                            |                                     |
| <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> |                                                                                                                                                                                                                                                                                        |                                     |
| <b>IV. CERTIFICATION</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                                                                                                        |                                     |
| Date of the Actual Completion of the International Search                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | Date of Mailing of this International Search Report                                                                                                                                                                                                                                    |                                     |
| 10 APRIL 1992                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 5 APR 1992                                                                                                                                                                                                                                                                             |                                     |
| International Searching Authority                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | Signature of Authorized Officer                                                                                                                                                                                                                                                        |                                     |
| EUR PEAN PATENT FFICE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | NAUCHE S.A.                                                                                                                                                                                                                                                                            |                                     |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |                                                                                                                                                                                                                                                                                                                                                                                                       |                       |
|----------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category *                                                                 | Citation of Document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                    | Relevant to Claim No. |
| A                                                                          | <p>JOURNAL OF NEUROCHEMISTRY<br/>vol. 46, no. 4, 1986, RAVEN PRESS, NEW YORK US<br/>pages 1058 - 1067;<br/>Urwyler, Stephan; Markstein, Rudolf:<br/>'Identification of dopamine "D3" and "D4"<br/>binding sites, labeled with [3H]2-amino-6,7-dihy<br/>droxy-1,2,3,4-tetrahydronaphthalene, as high<br/>agonist affinity states of the D1 and D2<br/>dopamine receptors, respectively'</p> <p>---</p> |                       |